

Dinucleoside polyphosphates: newly detected uremic compounds with an impact on leukocyte oxidative burst

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Abstract

Background. Dinucleoside polyphosphates (Np_nN) have patho-physiologic roles in cardiovascular disease and are newly detected uremic retention solutes. They were retrieved in human plasma, tissues and cells. Although their impact on several cell systems involved in vascular damage (endothelium, smooth muscle cells and thrombocytes) has been evaluated, their effect on different types of leukocytes has never been studied.

Methods. This study evaluates, for the first time, the impact of Np_nN on monocyte, granulocyte and lymphocyte oxidative burst activity at baseline and after stimulation with fMLP and PMA in whole blood. Ap_3A to Ap_6A were tested to investigate the effect of the number of phosphate groups on ROS production. The effect of the type of nucleoside was evaluated by comparing Ap_4G , Gp_4G , Up_4A and Ap_4A .

Results. This study demonstrated that especially lymphocytes are susceptible to Ap_nA . Depending on phosphate chain length different effects were observed. At baseline and with fMLP, Ap_4A , Ap_5A and Ap_6A enhanced lymphocyte free radical production. In addition, Ap_3A , Ap_4A and Ap_5A increased PMA-stimulated ROS production in lymphocytes. Monocytes and granulocytes parallel the lymphocyte response albeit with an inhibition of Ap_6A on granulocytes. Considering Np_nN with four phosphate groups, Up_4A showed the most important stimulatory effects on monocytes and Ap_4A on lymphocytes.

Conclusions. Np_nN mainly have a leukocyte activating impact, most significant for Ap_4A , considering phosphate chain length, and for Up_4A , considering the type of nucleosides. These results suggest that pro-inflammatory effects of Np_nN can contribute to the development of atherosclerosis, probably in the early stages of chronic kidney disease but their chemical composition affects their activity.

Keywords

Cardiovascular disease, Cell activation, Chronic kidney disease, Oxidative stress, Reactive oxygen species, uremic toxins

Short summary: The dinucleoside polyphosphates, a group of newly identified uremic retention solutes, were tested on the leukocyte oxidative burst activity. Both the effect of the number of phosphate groups as well as the type of nucleosides were evaluated. Up₄A and Ap₄A were considered the most important molecules in monocytes and lymphocytes and thus can contribute to the vascular damage seen in CKD.

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Introduction

In chronic kidney disease (CKD), the risk for premature death, primarily as a result of cardiovascular disease (CVD), is high and this risk increases early on in kidney failure [1,2]. Traditional risk factors insufficiently predict cardiovascular outcome in CKD; less traditional risk factors, like inflammation, endothelial dysfunction, oxidative stress, vascular calcification and malnutrition, seem to play an at least as important role [3-6].

When renal function deteriorates, a number of substances, normally excreted in the urine, are retained within the body. In 2003 a list of 90 known uremic compounds was published and several of these have the potential to induce vascular damage [7]. However, further identification of unknown compounds and the elucidation of pathophysiological mechanisms remains necessary to better understand the process of vessel damage in CKD and to develop specific therapeutic interventions [8-10].

The dinucleoside polyphosphates (Np_nN) are a newly identified group of compounds only recently added to the list of uremic retention solutes [11]. Np_nN contain two nucleosides interconnected by a variable number of phosphates; with a molecular weight between 800 and 1200 Dalton, they belong to the so-called “middle molecules” (molecular structures: see Figure 1) [12].

Specific members of this group have been detected in human plasma, platelets, neuronal cells, endothelial cells, adrenal glands and myocardial tissue [13-16]. In hemodialysis patients, the intracellular diadenosine polyphosphates (Ap_nA) are increased in platelets and released upon activation [17]. Also renal tubular cells release Ap_5A and Ap_6A and uridine tetraphosphate (Up_4A) and Up_4A is released by the endothelium as well [18].

The Np_nN play a role in vasoregulation, neurotransmission and cell signalling [19-21]. Related to the cardiovascular system, Np_nN have been shown to interfere with the function of thrombocytes, endothelium and smooth muscle cells (SMC) [16,19,22-24]. Despite the fact

that leukocytes are also key mediators in vessel damage, studies of Np_nN with these cells are scarce and fragmentary, both regarding the type of cell and the Np_nN variant. Leukocytes play an important role in the immune response. CKD is considered as a state of chronic inflammation characterized by a dual immune response; on the one hand, many CKD patients have a baseline status of inflammation, while on the other hand, their immune function upon stimulation is often suppressed, resulting in an increased susceptibility to infection [5,25].

The present study investigates the biological impact of a series of Np_nN on leukocyte function to evaluate their contribution to the uremic syndrome. Chronic inflammation in CKD is associated with oxidative stress and since leukocytes are an important source of reactive oxygen species (ROS) [26,27] the effect of the Np_nN was evaluated by measuring changes in leukocyte oxidative burst activity. The effect of the number of phosphate groups on leukocyte oxidative burst activity was studied by testing Ap_3A to Ap_6A , the subgroup of Np_nN on which the most extensive information is available. Since the most pronounced effects in this analysis were found for Ap_4A and in view of the recently demonstrated important vasoconstrictive effects of Up_4A [18], a second series of experiments was performed concentrating on known Np_nN with 4 phosphate groups but a varying type of nucleoside i.e. Ap_4A , adenosine guanosine tetraphosphate (Ap_4G), diguanosine tetraphosphate (Gp_4G) and Up_4A . Finally, the effect of a combination of Ap_4A and Up_4A was tested.

Materials and Methods

Reagents

High pressure liquid chromatography (HPLC) water (gradient grade) and acetonitrile (ACN) were purchased from Merck (Germany); all other substances were obtained from Sigma-Aldrich (Germany), unless otherwise specified.

Synthesis of Np_nN

The Np_nN tested in vitro were Ap_3A , Ap_4A , Ap_5A , Ap_6A , Ap_4G , Gp_4G and Up_4A . They were all synthesized following a protocol described by Ng and Orgel [28] and chromatographed according to a method by Jankowski et al. [29].

Briefly, depending on the type of Np_nN , adenosine 5'-polyphosphates, guanosine 5'-polyphosphates and/or uridine 5'-monophosphate were dissolved in water together with *N*-[2-hydroxyethyl]-piperazine-*N'*-2-ethanesulfonic acid, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide, and $MgCl_2$ and incubated at 37°C for 48 hours at pH 6.5.

Dinucleoside polyphosphates were concentrated on a C18 reversed-phase column (Supersphere 100 C18 endcapped, 100 x 2; 1mm, 4 μ m, 10 nm, Merck, Germany) using 40 mM aqueous triethylammonium acetate (TEAA) and were eluted with 30% acetonitrile in water. The lyophilized concentrate of the reversed-phase column was injected on two C18 reversed-phase columns connected in series (Supersphere, 300 x 8 mm, 4 μ m, Merck, Germany) and was chromatographed in the displacement mode by use of *n*-butanol (100 mM). The fractions containing Np_nN of the displacement-chromatography were lyophilized and each fraction was chromatographed with an anion-exchange column (column: UnoQ (Biorad), 100 x 10 μ m, 10 μ m, eluent A: 20 mM K_2HPO_4 ; eluent B: 20 mM K_2HPO_4 and 1 M NaCl; gradient: 0–10 min: 0–5% B; 10–115 min: 5–40% B; and 115–120 min: 40–100% B; flow rate: 1.0 ml/min; and UV absorption wavelength: 254 nm). The fractions of the anion-

exchange chromatography were desalted by HPLC reversed-phase C18 chromatography. The chromatographically isolated Np_nN were lyophilized and stored at -20°C [29].

Sample collection

The present protocol was approved by the local Ethics Committee after informed consent. Heparinized whole blood from healthy donors, not smoking and not taking any medication, was collected. Additionally, heparinized whole blood was taken from hemodialysis patients prior to the dialysis session.

In vitro effect of dinucleoside polyphosphates on the leukocyte oxidative burst

Experimental set-up

The present study was performed according to a standardized protocol for in vitro testing of uremic retention solutes described by Cohen et al. [30], generated after ample discussion among the experts of the European Uremic Toxin Work Group (EUTox). This monograph recommends to test the highest reported uremic concentration. It is of note however that current data on Np_nN concentrations are still limited. Up till now, only Jankowski et al. described an increased intracellular amount of Ap_nA in platelets and an increased release by platelets of hemodialysis patients upon activation. A maximum release of 154 ± 59 fg Ap_4A /platelet (range from 95 to 213 fg Ap_4A /platelet) was described [17] and since whole blood contains 4×10^5 platelets/ μl we calculated a mean local concentration of 61.6 ng Ap_4A / μl blood (range: 38.0 to 85.2 ng Ap_4A / μl blood) or 72.2 μM (range: 45.4 to 101.9 μM). In the in vitro set-up therefore a concentration of 100 μM was tested for each Np_nN which, based upon the current knowledge, rather reflects local concentrations of Np_nN after release from their storage cells than their plasma concentration. These values also correspond to the local concentrations reported by Ogilvie [31].

The lyophilized Np_nN were resuspended separately in saline (0.9% NaCl, Baxter) in a 10 times concentrated stock solution of 1 mM and stored at -20°C and upon testing they were diluted 1:10 in heparinized whole blood.

Oxidative burst

To evaluate the effect of the Np_nN on the production of reactive oxygen species (ROS) in leukocytes, the Bursttest (Phagoburst®) (Orpegen Pharma, Heidelberg, Germany) was applied, after a 10 minute incubation period at 37°C in the presence of the separate Np_nN, as described previously [32].

The oxidative burst activity of the leukocytes was measured at baseline, after moderate stimulation with N-formyl-Methionine-Leucine-Phenylalanine (fMLP, 0.83 µM) and after strong stimulation with Phorbol-12-Myristate-13-Acetate (PMA, 1.35 µM). Samples were analyzed with the FACScan® flow cytometer (Becton Dickinson). Experiments were repeated 8 (Ap_nA) or 6 (Np₄N) times.

FACScan Analysis

Using the CellQuest Pro™ software, 10,000 events were counted in every sample. Based on their light scatter properties monocytes, granulocytes and lymphocytes were gated separately. Within these gates the percentage of rhodamine-positive cells (%) was evaluated for the baseline and fMLP stimulated samples. The mean fluorescence intensity (MFI) per cell was considered as a measure for the oxidative burst activity after PMA stimulation.

Endotoxin concentration

To exclude that an effect due to lipopolysaccharide (LPS) contamination was observed, all experimental solutions causing a stimulation of the oxidative burst were checked by means of the Limulus Amebocyte Lysate (LAL) QCL-1000-test; a quantitative kinetic and

chromogenic assay (Cambrex Bio Science, Walkersville, MD, USA). The detection limit of this assay is 0.005 endotoxin units (EU)/ml.

Statistical analysis

Data are expressed as mean \pm SEM. Normality was checked with the Kolmogorov Smirnov – test in combination with descriptive statistics. Statistics were performed using a paired t-test for the in vitro data. A P-value of < 0.05 was considered significant.

Results

Effect of a varying number of phosphate groups on the leukocyte oxidative burst activity

Effects of Np_nN with a varying number of phosphate groups linking the two nucleoside moieties are described. To evaluate whether the number of phosphate groups plays a role in the effect of the Np_nN on leukocyte oxidative burst activity, four Ap_nA with 3 to 6 phosphate groups, were tested.

At baseline, 4.65 ± 0.10 % of the monocytes, 4.76 ± 0.07 % of the granulocytes and 2.63 ± 0.53 of the lymphocytes ($n = 8$) produced ROS. As presented in Figure 2A, incubation with Ap_4A and Ap_5A induced a significant rise ($P < 0.05$, $n = 8$) in free radical production in all three leukocyte types. For both monocytes and lymphocytes also Ap_6A resulted in a significantly increased percentage of rhodamine positive cells. Finally, for Ap_3A no significant effects were observed.

A moderate stimulation of the bursttest with fMLP resulted in a significant increase of the ROS production for monocytes and granulocytes, in the saline condition as well as in the presence of Ap_nA . However, only Ap_6A affected the fMLP induced ROS production in comparison to the saline condition in monocytes. The oxidative burst in lymphocytes remained unaffected by fMLP as previously demonstrated and the Ap_nA showed an enhanced activity versus saline which was comparable with effects observed in baseline cells (Figure 2B) [33].

After stimulation with PMA, 97.1 ± 1.51 % of the leukocytes produce ROS and therefore the MFI was evaluated for these samples. As demonstrated in Figure 2C, Ap_4A further enhanced the oxidative burst activity in monocytes, while Ap_6A inhibited it in granulocytes. The most significant effects were seen in the lymphocytes, where Ap_3A , Ap_4A and Ap_5A significantly enhanced free radical production.

The present data demonstrate that depending on the number of phosphate groups and the type of leukocyte, Ap_nA have a different impact on the oxidative burst activity at baseline and after stimulation. In general, they have a leukocyte activating impact even in combination with another activator. This effect is most prominent in lymphocytes.

Effect of varying the type of nucleoside

Based on the previous results, the number of phosphate groups was maintained constant at 4 in the second series of experiments, and the effect of the type of nucleosides on the oxidative burst response was studied by testing Ap_4A , Ap_4G , Gp_4G and Up_4A .

Figure 3A represents the oxidative burst activity at baseline (A) for monocytes, granulocytes and lymphocytes. Compared to Ap_4A , which was stimulatory for all cell types, for Ap_4G and Up_4A a stimulatory effect was observed in monocytes while Ap_4G and Gp_4G both inhibited the ROS production in granulocytes.

The monocytic fMLP stimulated oxidative burst activity was enhanced in the presence of Up_4A , while no effects on the fMLP stimulated bursttest in granulocytes were seen. None of the compounds with 4 phosphate groups, except for Ap_4A , showed an effect on lymphocytes, neither at baseline nor after fMLP stimulation (Figure 3B).

As shown in Figure 3C, Up_4A caused, in parallel with Ap_4A , a significant rise in free radical production in PMA stimulated monocytes and lymphocytes. No effect was seen on the PMA-stimulated granulocytes or after incubation with Ap_4G and Gp_4G .

By studying the effect of different types of nucleosides (Np_4N), it can be concluded that again different effects are observed in the different types of leukocytes under study. The most important effects are observed in the presence of Up_4A , especially in monocytes and with Ap_4A in lymphocytes. However, after PMA stimulation Up_4A induces a significantly higher response compared to Ap_4A in both cell types.

Effect of the combination Ap₄A and Up₄A

Because of their most prominent effects, the effect on leukocyte ROS production of a combination of Ap₄A and Up₄A was evaluated (each at 100μM). Although the above described results were confirmed in blood from healthy donors, no cumulative effect of the compounds could be demonstrated (data not shown).

In contrast, when the Ap₄A and Up₄A mixture was added to whole blood obtained from hemodialysis patients, collected just before start of hemodialysis, no significant effects on the ROS production could be observed (data not shown).

Endotoxin concentration

All solutions tested were checked for their endotoxin concentration by performing the LAL-test. None of them had a LPS concentration above 0.05 endotoxin units (EU)/ml, which corresponds to 4 pg LPS/ml, except for Ap₆A containing 0.26 EU/ml.

When testing LPS in the bursttest at this concentration, no effect was observed however. Therefore, it can be excluded that the effects observed are attributable to endotoxin contamination.

Discussion

The present study evaluated the role of the Np_nN as newly identified uremic retention solutes in CKD related inflammation and atherogenesis. To the best of our knowledge, it is the first time that the impact of different Np_nN on oxidative burst activity of several types of leukocytes was tested, at baseline as well as after stimulation. The effect of the number of phosphate groups was studied in the most studied subgroup of dinucleoside polyphosphates, the Ap_nA ($n = 3$ to 6). Based upon the results the effect of the type of nucleoside was studied on Np_nN with 4 phosphate groups but a varying type of nucleoside, i.e. Ap_4A , Ap_4G , Gp_4G , Up_4A . Also the effect of a combination of Ap_4A and Up_4A was tested.

Mainly a pro-inflammatory effect of the Np_nN on leukocytes was found. Depending on the number of phosphate groups, especially lymphocytes were susceptible to the Ap_nA , with Ap_4A inducing a marked stimulation of the oxidative burst activity in cells at baseline, but also after fMLP and after PMA stimulation (Figure 2). Considering the type of nucleoside, Up_4A was shown to exert the most significant stimulatory effects on the basal and fMLP-activated monocytes (Figure 3A and B) and after PMA stimulation in both monocytes and lymphocytes (Figure 3C). A combination of both Ap_4A and Up_4A revealed a comparable, but no cumulative effect on ROS production by normal leukocytes, whereas on uremic leukocytes no effect on ROS production could be observed.

Oxidative stress plays an important negative role in the cardiovascular outcome of the CKD patient and oxidative stress is present already in the early stages of CKD [27,34]. The present study learns that Np_nN , especially the ones containing 4 phosphate groups, may contribute to this pro-inflammatory status.

Also the type of nucleoside can be at the origin of diverse effects, as is described for vasoconstriction of arteries where one adenine seemed crucial and sufficient because of the equipotent effect of Ap_nA and Ap_nG , whereas in contrast Gp_nG were inactive [24]. In the

present study it was shown that the presences of adenosines and uridines have a stimulatory effect on lymphocytes and monocytes, whereas guanosines have an inhibitory effect on granulocytes.

The observation that an increase of Np_nN in an extreme uremic milieu does not induce additional ROS production by uremic leukocytes as was observed for normal leukocytes, leads to the assumption that the effects observed with healthy donor blood mirrors the importance of their effect in the early stages of CKD playing a role in the initial phase of the atherosclerotic process.

The observed inhibitory effect in the presence of Ap_6A and the G-containing Np_nN , can be related to the enhanced susceptibility of the CKD patient for infection [25]. Opposite effects related to the phosphate chain length in the Np_nN have already been demonstrated previously in vasoactivity or platelet aggregation [24,35].

The specificity of the nucleosides for the different leukocyte subpopulations is most probably due to variability in their purinergic P2 receptor expression patterns for Np_nN . There are two main families of P2 receptors, P2X and P2Y. P2X receptors form plasma membrane channels selective for monovalent and divalent ions and do not require intracellular messengers. P2Y, on the other hand are G-protein coupled receptors and upon activation inositol 1,4,5-triphosphate is generated and intracellular Ca^{2+} is released, followed by a Ca^{2+} influx from outside the cell [36]. Each of these receptors has a series of subtypes and as recently reviewed in hematopoietic cell lines, many of them are present in leukocytes [37]. Next, a series of interactions of ATP and ADP with P2Y receptors on leukocytes were described, during vascular injury [38]. The type of P2 receptors playing a role in the currently observed effects on ROS production can differ among the type of Np_nN tested, the cell type and the stimulus. The use of inhibitors like suramin, a P2 purinergic antagonist, α,β -methylene ATP, a P2X1 inhibitor or other selective antagonists should elucidate this in the future.

Data on the effects of Np_nN on leukocytes had been, up to now, limited to evaluation of the effect of some Ap_nA in neutrophils. It was found that Ap_3A and Ap_4A are able to prime the respiratory burst if followed by further fMLP stimulation in isolated neutrophils and this effect was Ca^{2+} -dependent with Np_nN concentrations above 50 μM . The priming effects appeared to be maximal when the Ap_nA were added 1 minute before fMLP and at 600-800 μM [39]. The same group also found that Ap_nA ($n = 3 - 6$) delayed neutrophil apoptosis [40,41]. They also suggested that neutrophils express P2 receptors with different binding affinities to mononucleotides and dinucleotides [42]. Vartanian et al. found that interferons induce an accumulation of Ap_3A in both a monocytic and a granulocytic human cell line due to an accumulation of the enzyme tryptophanyl-tRNA synthetase [43].

According to the standardized protocol for in vitro testing of uremic retention solutes developed by European uremic toxin workgroup (EUTox), the Np_nN were tested at their highest estimated uremic concentration [30]. Considering the still limited available data on concentrations of Np_nN in CKD, the Np_nN concentration was based upon the release of Ap_nA by thrombocytes from hemodialysis patients [17]. Np_nN are released by endothelial cells and platelets, which are both cell types playing a role in atherogenesis. Hence, it is likely that intravascularly leukocytes are locally exposed to similar Np_nN concentrations especially if the producing cells are activated, as is the case for thrombocytes and endothelium in uremia [44].

The present data were obtained by performing acute experiments, with an exposure time of only 10 minutes. In vivo, the chronic exposure in CKD is however continuous, especially in atherosclerotic lesions, which contain macrophages, lymphocytes as well as thrombocytes, and thus the damaging impact is conceivably more persistent [26]. Of note, another possibility that should be considered is that the damaging effect of Np_nN in vivo is attenuated by compensatory, regulatory and/or repairing mechanisms. Although such mechanisms certainly

are at play, it should be taken into account that the net in vivo result of these interactions still essentially is pro-inflammatory.

The definition/specification of Np_nN as uremic retention solutes is mainly based on the following observations; Np_nN are endogeneous compounds with a strong impact on physiologic and pathophysiologic processes in the cardiovascular system [21,22,45]; Np_nN are released by different cell types involved in atherosclerosis, the major cause of death in CKD (e.g. thrombocytes, endothelial cells) [15,18]; platelets from hemodialysis patients have an increased intracellular Ap_nA concentration and an increased release [17]; Up_4A plasma concentration is increased in juvenile hypertensives versus normotensives and Up_4A is described to affect glomerular filtration rate [22,45]. Uremic plasma concentrations in se have however rarely been reported. The quantification of Np_nN in CKD (stage 2-5) and in uremic patients before and after hemodialysis is currently under investigation in different ongoing studies. Based upon the increased release of Ap_nA from platelets from hemodialysis patients [17], local concentrations of Np_nN were calculated to rise up to 100 μM .

Based on their molecular weight ranging from 800 to 1200 Dalton, the Np_nN are classified as middle molecules. In addition, dinucleosides were shown to be protein bound [13]. Because of their physico-chemical characteristics Np_nN are expected to be difficult to remove by dialysis, bringing along the need for more advanced removal strategies such as on line hemodiafiltration, adsorption and/or pharmaceutical interventions in function of the involved patho-physiological mechanism [10,12].

In conclusion, this study describes for the first time the effect of a series of Np_nN on leukocyte oxidative burst activity in relation to uremia. Depending on the length of the phosphate chain, the type of nucleoside and the type of leukocyte, a different impact on the oxidative burst activity at baseline and after stimulation was observed. In general these data,

especially showing a pro-inflammatory effect, suggest that Np_nN are likely to be involved in the development of atherosclerosis, probably in the early stages of CKD.

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Conflict of interest statement

None declared

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Figure legends

Figure 1. Molecular structure of Np_4N : Ap_4A (A), Ap_4G (B), Gp_4G (C) and Up_4A (D)

Figure 2. Effect of the Ap_nA ($n = 3-6$) on the oxidative burst activity of monocytes, granulocytes and lymphocytes at baseline (A), after fMLP stimulation (B) and PMA stimulation (C). Data are expressed as the percentage rhodamine positive cells (%) at baseline and for the fMLP stimulus and as the mean fluorescence intensity (MFI) for the PMA stimulus. * $P < 0.05$, ** $P < 0.01$ versus saline; $n = 8$

Figure 3. Effect of the Np_4N on the oxidative burst activity of monocytes, granulocytes and lymphocytes at baseline (A), after fMLP stimulation (B) and PMA stimulation (C). Data are expressed as the percentage rhodamine positive cells (%) at baseline and for the fMLP stimulus and as the mean fluorescence intensity (MFI) for the PMA stimulus. * $P < 0.05$ versus saline; $n = 6$